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Turning up the green light

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Keywords

Fluorescent proteins, protein engineering, brain imaging, super-resolution microscopy

Abstract (50-word limit)

Fluorescent proteins have become indispensable tools in biological research for labeling cells and proteins and sensing their biochemical activity. By introducing ‘folding mutations’, Campbell *et al.* engineered a new green fluorescent protein variant with dramatically enhanced cellular brightness and stability, facilitating advanced cellular bio-imaging applications in neuroscience and beyond.

Main text (1000-word limit)

Progress in neuroscience research depends strongly on the development of ever more powerful cellular bio-imaging techniques. Not only are better microscopes with sharper optics needed for this, but also brighter dyes and more specific ways to label the structures and molecules of interest. Clearly, the rise of modern fluorescence microscopy has been paved by ground-breaking advances on both fronts.

This is true for multi-photon microscopy of brain circuits, which relies on the expression of bright fluorescent reporters in specific cell populations [1], as well as for super-resolution imaging of subcellular structures, where tunable photophysical properties of the fluorophores are critical for imaging beyond the diffraction barrier of light [2].

After the discovery of Green Fluorescent Protein (GFP) and early modifications to make it brighter and shine in more colors [3, 4], efforts have mostly concentrated on making fluorescent proteins (FP) smarter and more versatile. As a fruit, FPs can now serve as genetically encoded optical actuators (like the light-gated ion channels ChR2), and biosensors (like the calcium indicator GCaMP6), offering control over their expression (timing, cell-specificity, sub-cellular targeting) and spectral properties (like photo-activatable GFP and photo-convertible mEos4) [5, 6].

By contrast, the efforts to improve the basic photophysical and chemical properties of FPs seemed to have reached the end of the road of what is possible. However, limited brightness and stability of existing FPs continue to pose a major bottleneck in neuroscience research, especially for advanced 'photon-hungry' applications, where high spatial and/or temporal resolutions and long observational times are required.

In this context, Campbell *et al.* [7] have recently engineered a new fluorescent protein, called mGreenLantern (mGL), which gives much brighter signals inside cells in culture or in tissue than the standard bearers like EGFP, YFP, Clover and mNeonGreen, by up to six-fold. Notably, this feat was accomplished not by modifying the chromophore region of the FP, but rather by introducing combinations of mutations that are known to affect the way GFP folds,

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presumably increasing the fraction of well-folded, viable monomeric protein in a cellular context (see Figure).

Instead of screening random mutations or creating fusions with poorly folded proteins in their hunt for FP variants with improved folding properties, the scientists selected 11 mutations from among previously described super-folding and well-expressing GFP variants to create mGL. By quantitatively comparing transfected mammalian cells, the effective 'cellular brightness' was found to be highest for mGL, even though its 'molecular brightness' (defined as the extinction coefficient multiplied by the quantum yield) is actually not higher. Strengthening the conclusions, the outstanding brightness was observed in several different host organisms and by different imaging methods.

While the mGL basically has the same photophysical properties (excitation and emission spectrum) as its GFP relatives, it exhibits much greater resistance to thermal and chemical stress. When incubated in a denaturing solution, all other tested FPs went dark instantly except for mGL, which continued to emit signal after 10 min, demonstrating an improved thermodynamic stability that may partly explain its high cellular brightness.

Such improved stability is beneficial for expansion microscopy and optical clearing, where chemically fixed tissue gets increased in size or is rendered transparent, respectively, to improve resolution and contrast in fluorescence images. As both techniques expose FPs to harsh chemical conditions, immunohistochemistry is typically required to enhance the labeling. However, mGL preserves its brightness, saving a processing step, which is not only laborious and expensive, but also adds background noise to the images.

In addition, by monitoring the kinetics of maturation, they also established that mGL exhibits the fastest maturation, being about 200% faster than EGFP (see Figure). Gel filtration chromatography of the purified protein and monitoring of artefactual structure formation at the endoplasmic reticulum when expressed in cells confirmed the lack of oligomerization besides a higher solubility in cells.

Despite the presumed changes in tertiary protein structure caused by the 'folding mutations' that bestow all these remarkable benefits, mGL conserves excellent photostability and is still recognized by regular GFP antibodies, permitting immunoprecipitation or nanobody purification.

The tangible benefits of the improved properties are manifold for neuroscience applications. As shown in the paper, virally expressing mGL as a cellular volume-filler of neurons in mouse visual cortex lights up their long-range axonal projections into sub-cortical areas, unlike EYFP, which leaves the distal and thin neurites invisible, facilitating neural circuit tracing to map the 'connectome' of the brain in mouse and zebrafish model systems [8, 9].

Likewise, virally driven expression of mGL also outperforms EYFP in terms of speed, reducing the time before the labeling becomes visible, facilitating developmental studies, where long expression lags otherwise may defeat their purpose. The authors also show that mGL does a much better job than EGFP at visualizing dendritic spines by 2-photon microscopy *in vivo* or wide-field imaging in cell culture, which are bread-and-butter applications for neuroscientists working on the plasticity of brain circuits or molecular machinery of synapses.

The exceptional brightness of mGL may also prove valuable for live-cell STED microscopy, which can resolve nanoscale cellular morphology and organelles in living tissue samples and even *in vivo* [10]. However, its inherently small fluorescence detection volumes together along with notoriously fast bleaching can quickly lead to inacceptable signal-to-noise ratios. Unfortunately, mGL actually appears to bleach faster than Clover. Given that mGL is much brighter to begin with, it might still permit more extended time-lapse imaging with STED microscopy than what is currently possible with existing FPs.

Taken together, Campbell *et al.* delivered a substantially improved FP by pursuing an innovative protein engineering strategy based on 'folding mutations', which may hold

100 additional optimizations in store. Mainly brighter and more stable than its predecessors
101 without compromising on any of its other relevant properties, mGL is primed for rapid
102 adoption by the bio-imaging community and bound to facilitate a wide variety of challenging
103 bio-imaging applications, ranging from different *in vivo* and *ex vivo* imaging modalities to
104 animal models and organ systems.

105 Shall this new lantern shine the way towards discovery!

106 **Figure**

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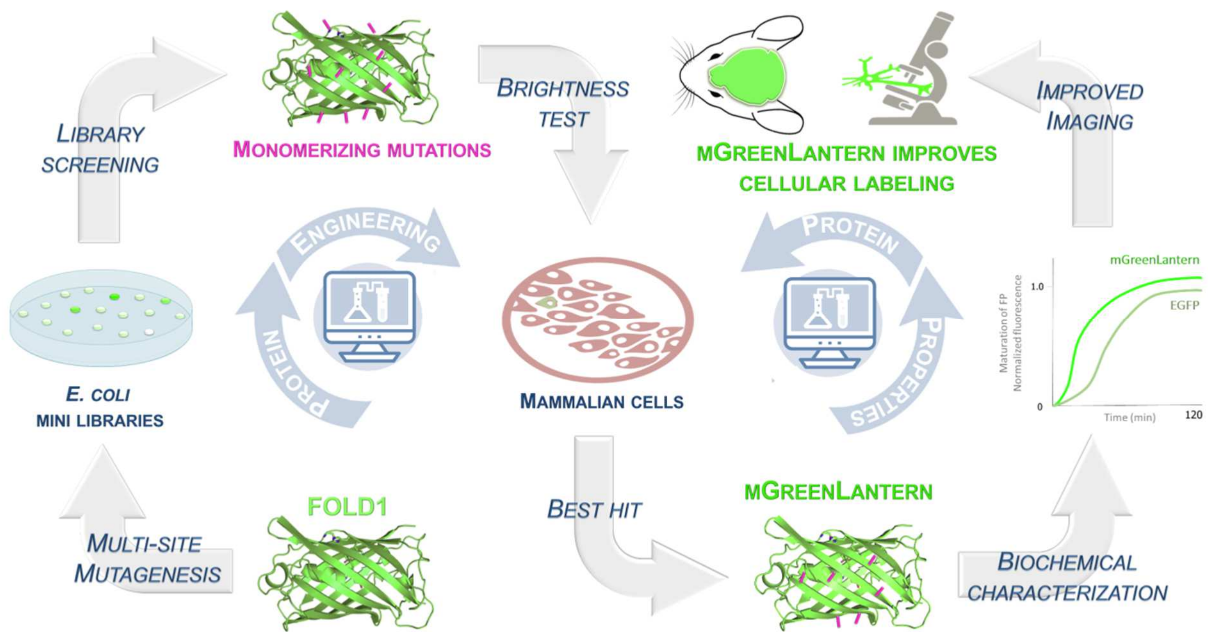

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109 **Engineering a new fluorescent protein (mGreenLantern)**

110 A combination of mutations was first introduced by multi-site mutagenesis from the FP
111 FOLD1. Monomerizing mutations based on screening of mini libraries were then selected.
112 Using brightness tests on mammalian cells, the brightest FP (called mGreenLantern, mGL)
113 was identified, which exhibited improved thermodynamic stability, faster maturation and no
114 signs of oligomerization. By pursuing a promising engineering strategy for enhancing the
115 performance of GFP-based biosensors, they developed a new FP with improved
116 photophysical and chemical properties, which facilitates advanced imaging applications in
117 neuroscience and cell biology.

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